AD_____

AWARD NUMBER DAMD17-94-J-4186

TITLE: The Genetic Analysis of the Role of Neu Differentiation Factor (Heregulin) in Neu-Induced Mammary Carcinomas in the Mouse

PRINCIPAL INVESTIGATOR: Archibald Perkins, M.D., Ph.D.

CONTRACTING ORGANIZATION: Yale University School of Medicine
New Haven, Connecticut 06520-8047

REPORT DATE: July 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Final (15 Jul 94 - 14 Jun 98)

4. TITLE AND SUBTITLE
The Genetic Analysis of the Role of Neu Differentiation Factor (Heregulin) in Neu-Induced Mammary Carcinomas in the Mouse

5. FUNDING NUMBERS
DAMD17-94-J-4186

6. AUTHOR(S)
Archibald Perkins, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Yale University School of Medicine
New Haven, Connecticut 06520-8047

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

We examined the role of neu differentiation factor (NDF) in mammary tumorigenesis using transgenic mice. NDF is a ligand for ErbB4, a member of the ErbB family of tyrosine kinase receptors, of which two other members (EGFR and NEU) have been implicated in human breast cancer. Transgenic mice expressing a chimeric ligand having an NDFβ-derived receptor binding domain within a TGFα backbone under an MMTV promote developed Harderian gland hyperplasias at high frequency. One mouse of 18 founders developed mammary carcinoma. Neither phenotype was transmissible to offspring. Other constructs, including NDFβ2A, and mutant derived from that isoform, failed to express at significant levels in transgenic mice, precluding further analysis. In a separate Task, we attempted to identify genes that cooperate with c-*neu* in mammary tumorigenesis by proviral tagging using MMTV that is passed from female C3H mice to offspring via their milk. However, the strain of C3H that we used has experienced phenotypic drift since its original derivation, and was found to have a very low incidence of mammary carcinoma for reasons that are not clear. We did find evidence suggesting the presence of modifier loci in the FVB and/or C3H strains that influence the latency of *neu*-induced mammary tumors.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

ASP Where copyrighted material is quoted, permission has been obtained to use such material.

ASP Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

ASP Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

ASP In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

NA For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

ASP In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

ASP In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

ASP In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

A S Pulcini 11/14/98

PI - Signature Date

TABLE OF CONTENTS

Introduction

Like most human cancer, breast cancer is the result of cumulative genetic alterations resulting in loss of growth control. The genes involved in this multistep process have not bee elucidated, but include p53, which is altered in more than 50% of cases, the Rb tumor suppressor gene, BRCA1 (5% of cases, mostly inherited, early onset), and the *neu/erbB-2/HER-2* gene in 20-30% of cases (Slamon, Clark et al. 1987). It is this last gene, *neu*, which is the focus of our research. *neu* is a member of a family of genes that encode receptor tyrosine kinases. Other family members include the epidermal growth factor receptor (*EGFR*), *erbB-3*, and *erbB-4*. Activated *neu* oncogenes are potent in transforming cells in culture and transgenic mice overexpressing either mutationally activated *or* normal *neu* in the mammary gland succumb to adenocarcinomas. The oncogenic effect of both activated and normal neu alleles was evident from whole animal studies (Muller, Sinn et al. 1988). When normal c-*neu* gene was driven by MMTV in transgenic animals, the tumors were focal adenocarcinomas surrounded by hyperplasia, and were not pregnancy dependent (Guy, Webster et al. 1992). Since the mode of c-*neu* participation in oncogenesis in humans is amplification rather than activating mutations at c-*neu*, this transgenic model more closely resembles the situation in humans, and the long latency and stochastic nature of the tumors emphasizes the need for other events in carcinogenesis.

The $p185^{neu}$ receptor encoded by *neu* is stimulated by two families of ligands: the EGF family, and the NDF family, which includes heregulin (Holmes 1992), also known as neu differentiation factor (NDF) (Wen, Peles et al. 1992). None of the EGF family members appears to bind directly to $p185^{neu}$, yet several can activate the receptor via transmodulation. This is believed to occur by binding of the ligand to a high affinity receptor (e.g., EGFR) that then physically associates with $p185^{neu}$ and heterodimerizes. The result of this physical association is phosphorylation and activation of $p185^{neu}$. Thus a variety of ligands can channel their signal through $p185^{neu}$, and the partners created depend on what other receptors are expressed in a given cell, and what ligands the cell is exposed to.

NDF is synthesized initially as a transmembrane glycoprotein with a 242 amino acid ectodomain that has an IgG-type motif and an EGF homology domain. The latter, contained in all members of the ErbB-binding ligand family, most likely functions in receptor binding. The transmembrane form, via proteolysis at a site near the ecto-/transmembrane domain junction, is likely to be the precursor for the released form, as is the case for other membrane-bound growth factors.

While data is now emerging concerning the role of NDF in mammary carcinogenesis, considerably more is known concerning the role played other ligands that act through ErbB family members. Transgenic mice overexpressing TGFα, either with promoters targeting mammary epithelium, or generalized promoters, display mammary epithelial hyperplasia and neoplasia that is often malignant, and often involves the terminal ducts and secretory alveoli (Matsui, Halter et al. 1990). Recent studies show a potent interaction between TGFα and c-*neu* overexpression in transgenic mice: By crossing the MMTV-c-*neu* transgenics with MMTV-TGFα mice, a strong cooperativity was found, resulting in rapid hyperplasia and milk production (Muller, pers. comm). Clearly TGFα has a mitogenic, growth-stimulatory role in breast development and in mammary carcinogenesis. The role of NDFs is unclear, but given the finding that it can promote differentiation and growth cessation in cultured mammary epithelial cells, it may act antagonistically to TG. It is the goal of these studies to explore the role of NDFs in mammary carcinogenesis in whole animals using genetic approaches.

Body

Task 1. Overexpression of NDF: Transgenics. *neu* is one of the few genes clearly implicated in the development of human mammary tumors. In addition, TGFα, a ligand for a related growth factor receptor, EGFR, can stimulate $p185^{neu}$ activity via transmodulation, and can also play a stimulatory role in mammary carcinogenesis. We hypothesize that NDF, a putative ligand for $p185^{neu}$ that can stimulate its activity, but which induces the differentiation of mammary cell lines, plays an important role in mammary tumors, especially those in which *neu* also has a causative role. To address this hypothesis, we created transgenic mice with overexpression of NDF or NDF-derived chimeric constructs targeted to the mammary gland.

In collaboration with Dr. Neal Copeland of the NCI-Frederick, a series of transgenic mice were created to assess the oncogenic potential of NDF in the mammary gland. To this end, we made transgenic mice with NDF under transcriptional control of MMTV (14 founders from 144 potential founder mice screened) and under control of the whey acidic promoter (WAP) (18 founders from 100 potential founders). For this experiment, we utilized the β2A isoform of NDF. As discussed above, this isoform has the β-type EGF homology domain which binds to the ErbB4 receptor with higher affinity than the α isoform (Wen, Suggs et al. 1994), and the A isoforms contain the longest cytoplasmic domain. We chose this domain since it stimulates higher tyrosine kinase activity than the α isoforms, and because it was thought that the longer cytoplasmic tail may have some important role.

A representative ethidium bromide-stained agarose gel electrophoretic analysis of the genotyping of these mice is shown in Figure 1A. Detection of the transgene was done by polymerase chain reaction (PCR) using primers in the NDF transgene.

We have analyzed these mice for expression, and were disappointed to find that the level of stable transcripts in lactating or late pregnancy mammary glands was very low (Figure 1B (all lanes) and Figure 2A lanes 12, 14, and 15 for MMTV-NDFβ2A; Figure 2A, lanes 1-10 for WAP-NDFβ2A), especially when compared with the level obtained with the WAP promoter driving other genes (Li, Rosen et al. 1997), or the MMTV promoter driving TGFα (Matsui, Halter et al. 1990). Nonetheless, we aged a cohort of MMTV-NDFβ2A and WAP-β2A transgenic mice for 18 months, and found no significant pathology in the 29 mice analyzed.

In an effort to accelerate what might have been a weak oncogenic stimulus, we treated a cohort of MMTV-NDFβ2A transgenic mice with the mammary carcinogen dimethybenzanthracene (DMBA) in an effort to obtain a mammary tumor with this transgene for two reasons: one is to see if transgene expression increases in the tumor (as is often the case), and if so, to have mammary carcinoma cell lines that express NDFβ2A for future study. Seven transgenic mice were treated with DMBA and five of these (4466, 4467, 4473, 4481, and 4483) developed mammary carcinomas. Representative histopathology is shown in the photomicrographs in Figure 1, panels C, D, E, and F. C and E are high-power photomicrographs of tumors in mice 4481 and 4483, respectively, which arose with latency of 159 and 163 days, respectively. Mouse 4481 also had a metastasis of the mammary tumor to the liver (Figure 1, panel D). The histopathology of the tumors that arose in the MMTV-NDFβ2A transgenic mice was a mammary adenocarcinoma, very similar to that seen in MMTV-c-neu transgenic mice (Li, Rosen et al. 1997): tightly packed carcinoma cells with moderate stroma and areas of central necrosis, and without any squamous

differentiation. In contrast, nontransgenic mice treated with DMBA typically develop adenoacanthomas of the mammary gland, which characteristically exhibit abundant squamous differentiation (e.g., mammary tumor of DMBA-treated nontransgenic mouse, Figure 1 panel F; arrow indicates area of squamous differentiation). Of note, while the histopathology appeared somewhat different in the MMTV-NDFβ2a transgenic mice compared with control mice, the latency was not appreciably shorter. Thus, while the transgene may have had an effect on tumor histology, it had no apparent effect on tumor latency. The mammary tumors that arose in these mice were explanted into culture and frozen for further analysis.

We have also made several other types of transgenics. As discussed above, NDF is produced as a transmembrane protein that is later cleaved at a specific site on the ectodomain to release a soluble ligand molecule. This is similar to the situation with stem cell factor (SCF, also known as Steel factor or mast cell growth factor). With this factor, it has been shown that the membrane-bound and soluble forms have different activities, and are probably both essential in mouse development. We wondered if a form of NDF that could not be cleaved would have higher activity than one that can be cleaved. To test this, we created transgenic mice that contain MMTV-NDFβ2A with a point mutation in the proteolytic cleavage site that is used to generate the soluble form. Unfortunately, expression of this transgene, called MMTV-mtNDFβ2A, could not be detected by Northern blot of total RNA (Figure 2A, lanes 11, 13, 20-24). Transgene expression could only be detected by reverse transcription-PCR, followed by Southern blotting (data not shown). Nonetheless, the founders (no offspring) were maintained in mating, to determine susceptibility to mammary carcinomas. Out of fourteen mice aged to 18 months, none developed mammary carcinomas or other lesions of significance.

We were concerned about the problem of low expression of the transgene, and approached this in several ways. One is that we obtained MMTV expression vectors from two other investigators (Phil Leder and Paul Jolicoeur), and constructed MMTV-NDFβ2A plasmids with these. We have transfected these into tissue culture cells and induced with dexamethasone (the MMTV LTR is responsive to dexamethasone). We compared the level of expression of these constructs to that seen with our transgenic construct, which was made with a vector from Robert Coffey. There was no significant difference in the level of expression between the different plasmids: all expressed at a fairly low level (data not shown).

These results are consistent with the notion that high level expression of NDF may be detrimental to the cell or the mouse, and is selected against. To test this, created and tested additional DNA constructs in transgenic mice. We knew that the MMTV-TGFα plasmid from R. Coffey worked well in transgenics and in cell culture, so it is likely that there is no selection against the expression of TGFα. Since the receptor specificity of the ligand is determined by the EGF homology domain, we thought we could change the specificity of the MMTV-TGFα ligand by replacing its EGF homology domain with that from NDF (the β–type EGF homology domain). It was postulated that this chimeric protein, called TNT (for TGFα-NDF-TGFα), would be expressed at higher levels in transgenic mice than NDFβ2A, yet would have the same biologic activity of NDFβ, at least in terms of EGFR family signaling.

Of eighteen female MMTV-TNT founders, ten were examined for expression by Northern blot analysis of mammary tissue biopsies, using a radiolabeled probe for the transgene (Figure 2B). Two were found to be positive: mouse 4330 and 4352 (Figure 2B, lane 5 from the left on the top panel, and lane 2 from the left on the bottom panel, respectively; faint but positive signal is denoted by dot). All eighteen founders were aged for tumors, and within three months, six exhibited

unilateral exophthalmos, which eventually became bilateral, resulting from progressive enlargement of the Harderian gland. This is a tubuloalveolar gland located within the orbit of many mammalian species though it is absent in primates. These were diagnosed histopathologically as hyperplastic adenomas. These lesions were noninvasive and nonmetastatic. RT-PCR analysis revealed that the transgene mRNA was present in the Harderian glands of affected animals but not in unaffected animals (data not shown). Unfortunately, in none of the dozens of offspring from these mice that were observed (for up to a year), did any Harderian gland adenomas develop. The reason for this nontransmission of phenotype is not clear.

It has been previously shown that MMTV-NDFβ2 expression leads to Harderian gland hyperplasia (Krane and Leder 1996): In the course of these studies, a report has been published describing the phenotype of transgenic mice having a MMTV-NDFβ2C transgene. (This NDF cDNA was cloned from a mouse mammary tumor, indicating that this isoform can be expressed in mammary tumors) (Krane and Leder 1996). The report also describes a persistence of the terminal end buds in the mammary glands of virgin transgenic mice, and the presence of mammary gland adenocarcinomas in older mice.

While the report of Krane et al (Krane and Leder 1996) has diminished the novelty of our transgenic experiments, they suggest that NDF can play a role in mammary carcinomas in the mouse. Indeed, in one of our MMTV-TNT transgenic founder mice (No. 4395), an adenocarcinoma of the mammary gland arose (Figure 2C-E), which had a histologic appearance similar to the adenocarcinomas that arose in the DMBA-treated MMTV-transgenic mice (Figure 1C, E) and to those that arise in MMTV-c-*neu* transgenic mice (Li, Rosen et al. 1997). This tumor was transplantable in nude mice (Figure 2F), and was established in tissue culture and frozen in a viable state for further growth and expansion. Unfortunately, like the Harderian gland adenoma phenotype, this susceptibility to mammary tumors was not transmissible in any of the offspring of this mouse (over ten observed), aged over a year.

The reciprocal construct, MMTV-NTN was also made and introduced into mouse zygotes for the derivation of transgenic mice. Sixteen transgenic female mice were identified, none of which were found to express the transgene. These mice were aged to 18 months, and none developed any noteworthy pathology.

Task 2. Targeted deletion of NDF via homologous recombination. In these experiments, we attempted to take another approach to testing the same hypothesis that NDF plays an important role in mammary development and neoplasia. If this hypothesis is true, then deletion of the gene encoding NDF should have effects on either mammary gland development or neoplasia, or both. It turns out that the creation of a null allele at the NDF locus is lethal in the homozygous state (Kramer, Bucay et al. 1996; Meyer and Birchmeier 1996), and thus a null allele is uninformative in terms of the effect of NDF on mammary carcinoma. Thus, we considered creating more discrete mutations in the NDF gene via homologous recombination in embryonic stem cells, with the goal of creating "hypomorphic" alleles of NDF. The goal was to create mutations that are partially functional rather than null, and thus may give viability and an informative phenotype. To this end, we identified four different bacterial artificial chromosome (BAC) clones that hybridize with a probe for NDF, and Southern blot analysis of one of these (BAC 598K20) is shown in Figure 3, panel A). These were obtained from a mouse (strain 129) BAC library available from Research Genetics Inc. BAC clones are typically 100 to 150 kilobases in length, and are thus likely to contain the majority of the NDF gene. In collaboration with Dr. Frank Jones, we had intended to specifically knock out the exon encoding the alpha variant EGF homology domain, which appears to be expressed preferentially in the mammary tissue. We had hoped that homozygous deletion of this variant (while retaining the expression of the beta isoforms, which are expressed predominantly in brain) would result in a mouse that is viable and yet lacks expression of the mammary-specific isoform of NDF. This would have allowed us to determine the specific role of

NDF in the mammary tissue. However, we have recently received a personal communication from Dr. C. Birchmeier, who stated that her lab has performed a knockout of the exon encoding the alpha EGF homology domain, and that there is no discernable phenotype in the resultant mouse (C. Birchmeier, personal communication through Dr. Frank Jones).

Task 3 Identification of protooncogenes that can cooperate with *neu*. It is clear from the studies of Muller and coworkers (Guy, Webster et al. 1992) that *neu* does not act alone in the generation of mammary carcinomas in transgenic mice. The long latency (5-8 months) and the solitary, stochastic nature of the tumors argues that other factors are necessary in the disease process. We thus hypothesized that while *neu* is an important oncogene in mammary tumorigenesis, other genes are involved. We proposed to identify what these other genetic factors are by retroviral mutagenesis and proviral tagging. This is was attempted by infection of transgenic MMTV-c*neu* mice with mouse mammary tumor virus (MMTV). We expected that infection of transgenic mice with the virus would cause an acceleration of tumorigenesis: a shortening of tumor latency, due to the activation of cellular genes that can cooperate with c*neu* in the development of tumors. The presence of the proviral tag in *cis* to the implicated oncogene would enable us to molecularly clone and characterize them.

To perform the experiment correctly, we backcrossed the MMTV-c*neu* transgene onto the C3H background for five generations, so that the genetic background will be essentially identical to C3H, the high mammary carcinoma strain that carries MMTV. After we had completed with this phase of the project, and had begun to age mice that had both the transgene and MMTV to look for acceleration of tumorigenesis, we received notification from the Jackson Labs that the strain of C3H that we had employed, namely the C3H/HeOuJ strain, no longer had the high incidence of mammary cancer that had been reported previously (Outzen, Corrow et al. 1985). Nonetheless, as we had already generated the cohorts of mice, we aged these for tumors. Concomitantly, we ordered C3H/N MTV+ mice from Clarence Reeders at NIH-FCRDC, and are aging a new cohort of mice generated from this cross.

Four groups of mice were followed. Group 1 were offspring of C3H/HeOuJ mothers that had inherited the transgene (Neu+MMTV); Group 2 were transgene positive offspring that were MMTV negative (sired by C3H males; Neu alone); Group 3 were offspring of C3H females without the transgene (MMTV alone); Group 4 were negative for both virus and Neu transgene. Mice were kept pregnant and lactating.

Mice in the four groups were followed for up to 18 months, checking each week for the presence of mammary tumors by palpation. Time of tumor onset was noted, and mice were sacrificed and necropsied when moribund or at 18 months, whichever was sooner.

We were able to make two observations in this experiment. One is that we confirmed that the C3H/HeOuJ strain has a relatively low incidence of mammary cancers. Of the 23 mice that were followed, only six (26%) developed mammary tumors, with an average latency of 383 days. This compares with an incidence of nearly 100% and a median latency of 217 d reported by Outzen et al in 1985 (Outzen, Corrow et al. 1985). The reason for this is not clear, although investigators at the Jackson Labs have investigated numerous possibilities. They have confirmed that there is virus present in milk from 100% of C3H/HeOuJ mice. They suspect that there may be attenuation of the milk-transmitted MMTV and a mutational change at the *Lps* locus. This change in phenotype has had a negative effect on our ability to carry out the Task as originally planned.

The second observation is that the latency of tumorigenesis due to the *Neu* transgene is dramatically delayed in the C3H/HeOuJ background. The median latency for tumor onset on the FVB background was 230 days, while the same transgene on the C3H/HeOuJ background was 314 days when the mice were sired by a male C3H/HeOuJ (MMTV negative), and 338 days when sired by a C3H/HeOuJ female (MMTV positive).

This is an important and unexpected finding and is likely due to the presence of mammary tumor susceptibility genes in the FVB background or resistance genes in the C3H/HeOuJ background. To test this, one could perform additional crosses between the two strains to analyze the mode of inheritance the delayed cancer phenotype. This could lead ultimately to the identification of genes that can modify the incidence of mammary carcinomas that are induced by a known oncogene, c-Neu. Such cancer modifier loci are of particular interest (e.g., (Drinkwater and Bennett 1991; MacPhee, Chepenik et al. 1995)) due to their importance in identifying groups of patients that have a higher or lower risk of cancer.

Despite the lack of an acceleration of tumorigenesis in MMTV-positive mice carrying the c-*neu* transgene relative to nonviremic transgenic mice, we decided to proceed with the genetic analysis of these tumors. DNA was isolated from 36 tumors, was digested with EcoR1, and was analyzed by Southern blot for the presence of somatically acquired proviruses. In our initial experiment (Figure 3, panel C), we have obtained evidence for proviral insertion in some of the tumors, evident by the presence of hybridizing bands that appear in addition to the endogenous proviral bands (e.g., compare lanes 35 with 36 in Figure 3, panel C). However, some tumors that arose in females that suckled apparently virus-positive dams do not show obvious evidence of clonal, somatically-acquired proviruses (e.g., lane 37). These analyses are preliminary: other restriction enzymes will need to be used, in order to reach more definitive conclusions concerning the MMTV status of these tumors. It appears, however, that certain tumors have acquired proviruses. Further analysis of the sites of insertion may yield some interesting conclusions.

In the period since setting up this experiment with the C3H/HeOuJ strain, we have learned that C3H mice available from NIH, specifically from Clarence Reeders at Frederick, MD, have a high incidence of MMTV-induced mammary tumors. We have obtained these mice, and are now crossing the C3H/MTV+ females with male mice transgenic for *neu*. Having been suckled on the C3H female, these mice should be viremic and will be developing mammary tumors. With this cross, we will hope to see an acceleration of tumorigenesis when both MMTV and *neu* are present, relative to either agent alone. These tumors will then be used to isolate retroviral insertion sites with the goal of identifying cooperating oncogenes.

Relationship to Statement of Work (SOW).

Task 1. Overexpression of NDF: Transgenics.

a. Construction of vectors: Done

b. Microinjection/Analysis: Done

c. Expansion of strain: Done

d. Aging for Tumors: Done

Task 2. Knockout of *Ndf*:

a. Creation of construct: Not complete.

Task 3. Identification of protooncogenes that can cooperate with c-*neu*.

a. Backcross toC3H: Done

b. Aging of mice for tumors: For the cross with C3H/HeOuJ, this is complete; for C3H/N, this is still ongoing.

c. Analysis of tumors and cloning of proviral insertion sites: Ongoing.

Please note that the SOW given above is what was proposed in the body of the Grant Application. However, in the SOW listed at the end of the Proposal (p24), there is another Task, specifically, to overexpress MDF via murine mammary fat pad implantation studies (listed as Task 2, which made the Task 2 of this report Task 3, and the Task 3 of this report Task 4).

Discussion.

The work we have pursued was aimed at understanding the basis of the latency in tumorigenesis seen in MMTV-c-*neu* transgenic mice. We specifically sought to determine the role of Neu differentiation factor (NDF), also known as Neuregulin or Heregulin, in mammary tumorigenesis. This was approached in Task 1, where we created a series of transgenic mice designed to overexpress the b2A isoform of NDF in the mammary epithelium, using the MMTV or whey acidic protein (WAP) promoter sequences to drive expression. In all, five different constructs were used to create transgenic mice, and for each, numerous founders (between eight and 15) were analyzed for expression. We were beset with a problem of low expression of the transgenes, with both the MMTV and the WAP promoters, and this precluded us from pursuing our hypothesis in an efficient and straightforward manner: in neither the MMTV-NDFβ2A nor the WAP-NDFβ2A mice did we see a significant phenotype in the mammary gland. Whether this was due to low expression or was the inherent low biologic activity of the ligand NDF was at first not clear. However, further work from our lab and that of others (Krane and Leder 1996), it became apparent that low level expression was a problem. To address this problem, we took several approaches, none of which was entirely successful. We tried using MMTV-transgene constructs from different labs (Paul Jolicoeur and Phil Leder), and tested these in cell culture with and without dexamethasone stimulation, but we found no significant improvement over the plasmid vector used for the transgenics, which originated in the lab of Robert Coffey.

We also tried putting the critical region of NDF, that which interacts with the ErbB4 receptor (the EGF homology domain) and implanted this into a construct that we knew was functional in transgenics (MMTV-TGFα), and found only modest improvement in expression. With this construct, termed MMTV-TNT, we did see a significant phenotype: within three months, six of the eighteen founder mice had developed hyperplasia of the Harderian gland, a phenotype that is occasionally seen in transgenic mice, especially those that express oncogenes under mammary-specific promoters. Furthermore, one of the MMTV-TNT transgenic founders, No. 4395, developed an adenocarcinoma of the mammary gland (Figure 3C). With this phenotype, we were essentially able to confirm the results of Krane and Leder (Krane and Leder 1996), that expression of the beta isoform of NDF in transgenic mice under the MMTV promoter leads to increased susceptibility to Harderian gland adenomas and mammary carcinomas (although the latter phenotype was seen in only one of eighteen founders). We experienced a serious technical problem, however, that has precluded further progress on this Task: neither the Harderian gland adenoma phenotype nor the mammary carcinoma phenotype transmitted from the founders to the offspring, despite transmission of the transgene. This technical hurdle stymied our ability to examine the potential interaction between NDF and c-*neu* in mammary tumorigenesis. Nonetheless, we did derive an explanted mammary tumor from the 4395 mouse that grows well in both nude mice and in tissue culture, and can provide a basis for further study.

In Task 2, we sought to examine the in vivo role of NDF by making a targeted mutation at the locus. In essence, to achieve this proposed endeavor ahead of the competition was beyond our capability and resources: several groups were able to achieve an NDF knockout, and to show an early lethality of the homozygous null embryos (Kramer, Bucay et al. 1996; Meyer and Birchmeier 1996). We assumed a fallback position of making more subtle knock-in mutations in NDF, which

we hoped would more clearly define the mammary-specific role of the gene. Unfortunately, while we were able to clone out four BACs for NDF, providing the DNA substrate for the knock-in construction, we were not able to finalize this. We have recently heard that the lab of Dr. C. Birchmeier has been able to achieve some of these mutations, but to date, none of them has been revealing concerning the mammary-specific function of NDF.

In Task 3 we attempted to identify genes that can cooperate in the development of mammary tumors in transgenic mice that express MMTV-c-*neu*. This was done using MMTV proviral tagging of cooperating oncogenes. Importantly we found that the C3H/HeOuJ strain had a much lower incidence and longer latency for mammary tumors than previously reported (Outzen, Corrow et al. 1985). This has precluded our ability to identify cooperating oncogenes by proviral tagging mutagenesis as originally planned. We did find, however, a marked lengthening in the latency of c-*neu*-induced mammary tumors due to the C3H/HeOuJ background, indicating the presence of modifier loci in these strains that affect the susceptibility to mammary carcinomas caused by c-*neu*. This would be an interesting area for future investigation. In ongoing studies, we are crossing *neu* trangenic males with C3H/MTV+ females obtained from NIH, which succumb at high frequency to mammary tumors. We expect that analysis of the offspring of this cross will reveal an acceleration of tumorigenesis due to MMTV, and will allow the isolation of cooperating oncogenes. These studies are ongoing. We are optimistic about the outcome of these experiments. We have recently had success at cloning proviral insertion sites by a technique called inverse PCR. This allows the rapid cloning of proviral insertions using PCR technology. This, combined with rapid mapping of genes in the mouse genome afforded by radiation hybrid mapping, should allow us to quickly assess the spectrum of proviral insertions in these tumors.

In summary, our studies have sought to delineate a role for NDF in mammary tumorigenesis. On the basis of our studies and those of others (Krane and Leder 1996), we can conclude that the role of NDF in this process is fairly modest relative to that of other ligands of the ErbB family of receptors, particularly TGFα and EGF. We presume that in the MMTV-TNT-induced mammary tumor in mouse 4395, an autocrine loop of pro-growth signaling has been established. We can test this in cell culture, since we have an explanted cell line from this mouse established. This may prove to be a valuable resource for examining ErbB4 and ErbB3 signaling, and the changes in gene expression that are due to the activation of these receptors. This will be the focus of future studies in our lab.

References

Drinkwater, N. R. and L. M. Bennett (1991). "Genetic control of carcinogenesis in experimental animals." Prog Exp Tumor Res **33**: 1.

Guy, S. T., M. A. Webster, et al. (1992). "Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease." Proc Natl Acad Sci, USA **89**: 10578-10582.

Holmes, W. (1992). Science **256**: 1205-1210.

Kramer, R., N. Bucay, et al. (1996). "Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development." Proc Natl Acad Sci USA **93**: 4833-4939.

Krane, I. and P. Leder (1996). "NDF/heregulin induces persistence of terminal end buds and adenocarcinomas in the mammary glands of transgenic mice." Oncogene **12**: 1781-1788.

Li, B., J. Rosen, et al. (1997). "*Neu/ErbB2* cooperates with p53 -72H during murine mammary tumorigenesis in transgenic mice." Mol Cell Biol **17**: 3155-3163.

MacPhee, M., K. Chepenik, et al. (1995). "The secretory phospholipase A2 gene is a candidate for the *Mom1* locus, a major modifier of *Apc-min*-induced intestinal neoplasia." Cell **81**: 957-966.

Matsui, Y., S. Halter, et al. (1990). "Development of mammary hyperplasia and neoplasia in MMTV-TGFa trandgenic mice." Cell **61**: 1147-1155.

Meyer, D. and C. Birchmeier (1996). "Multiple essential functions of neuregulin in development." Nature **378**: 386-390.

Muller, W. J., E. Sinn, et al. (1988). "Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene." Cell **54**: 105-115.

Outzen, H., D. Corrow, et al. (1985). "Attenuation of exogenous murine mammary tumor virus virulence in the C3H/HeJ mouse substrain bearing the *Lps* mutation." J Natl Cancer Inst **75**: 917-923.

Slamon, D. J., G. M. Clark, et al. (1987). "Human breast cancer: correlation of relapse and survival with amplification of the HER-2/Neu oncogene." Science **235**: 177-182.

Wen, D., E. Peles, et al. (1992). "Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit." Cell **69**: 559-572.

Wen, D., S. Suggs, et al. (1994). "Structural and functional aspects of the multiplicity of Neu differentiation factors." Mol Cell Biol **14**: 1909-1919.

Bibliography of Publications from this Grant:

In preparation

Huynh, LN, Li, B, Zelazny, ET, and Perkins, AS. Identification of MMTV proviral integration sites in MMTV-induced tumors in c-*neu* transgenic mice. In preparation.

List of Personnel Receiving Pay from this Effort

Baolin Li

Edward Zelazny

Dongxian Yue

Figure Legends

1A. Representative PCR analysis of MMTV-NDFβ2A transgenic mice, using primers in the NDF gene. "N" denotes the migration of the NDF-specific product, while "B" is the β-casein positive control for the PCR reaction. Positive reactions were obtained in lanes 2, 5, 12, 13, 14, and 18; which represent mice numbers 2524, 2555, 2609, 2612, 2622, and 2652, respectively.

1B. Northern analysis of total RNA (30 μg/lane) from mammary glands of MMTV-NDFβ2A transgenic mice for NDF transcripts. Shown is analysis of RNAs from the following mice: Lane 1, 2518; lane 2, 2540; 3, 2547; 4, 2577; 5, 2585; 6, 2590; 7, 2600; 8, 2604; 9, 2604; 10, 2609; 11, 2635; 12, 2671; 13, 2672; 14, 2674; 15, 2690; 16, 2697; 17, 2707; 18, 2718; 19, 2743; 20, 2746. Hybridization is with a radiolabeled fragment of NDF, and exposure is for 10 days. Ethidium bromide staining of the agarose gel prior to blotting indicated approximately equal loading in each lane, and negligible degradation of the RNA (data not shown). Weak bands of hybridization are seen in lanes 1, 3, and 5; the intense hybridization in lane 14 is likely from DNA contamination.

1C-F: Photomicrographs of H+E-stained section of mouse mammary tumors arising in DMBA-treated mice. Panel C: primary tumor in mouse 4481, MMTV-NDFβ2a transgenic; panel D: liver metastasis in mouse 4481; panel E: primary tumor in mouse 4483, MMTV-NDFβ2a transgenic; panel F: primary tumor in control nontransgenic mouse.

Figure 2. Panel A. Northern blot analysis of NDF transgene expression in transgenic mice carrying either wildtype or mutant forms of NDFβ2A. Analysis of WAP-NDFβ2A mice is shown in lanes 1-10, MMTV-mtNDFβ2A in lanes 11, 13, 20-24, MMTV-NDFβ2A in lanes 12, 14, and 15; MMTV-NTN in lanes 16,-19, 25. As controls, RNA from fibroblasts transfected with CMV-NDFβ2A or CMV-mtNDFβ2A are analyzed in lanes 26 and 27, respectively. 30 μg of total RNA was loaded per lane. Hybridization is with an "NTN" probe, derived from the NTN construct; exposure is for 15 days. Ethidium bromide staining of the agarose gel prior to blotting indicated approximately equal loading in each lane, and negligible degradation of the RNA (data not shown).

Panel B. Northern blot analysis of TNT transgene expression in the mammary glands of transgenic mice. 30 ug of total RNA is analyzed in each lane, from the mice designated at the top of each lane. Two panels are shown: a top panel with mouse no.'s 4270, 4275, 4291, 4292, 4330, 4335, 4345; and a bottom panel with mouse no's 4351, 4352, 4362, 4367, 4395, and 4452. The dots denote the presence of very faint transcript bands, present in mice 4330 and 4352.

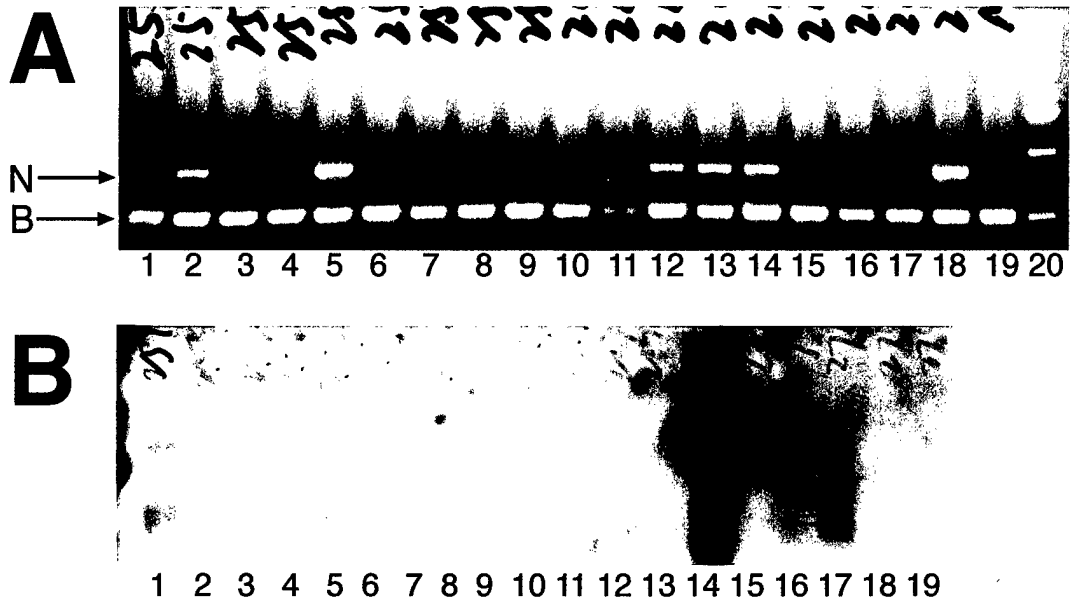
Panel C: Photograph of MMTV-TNT transgenic mouse no. 4395, bearing a large mammary tumor with superficial hair loss and erosion.

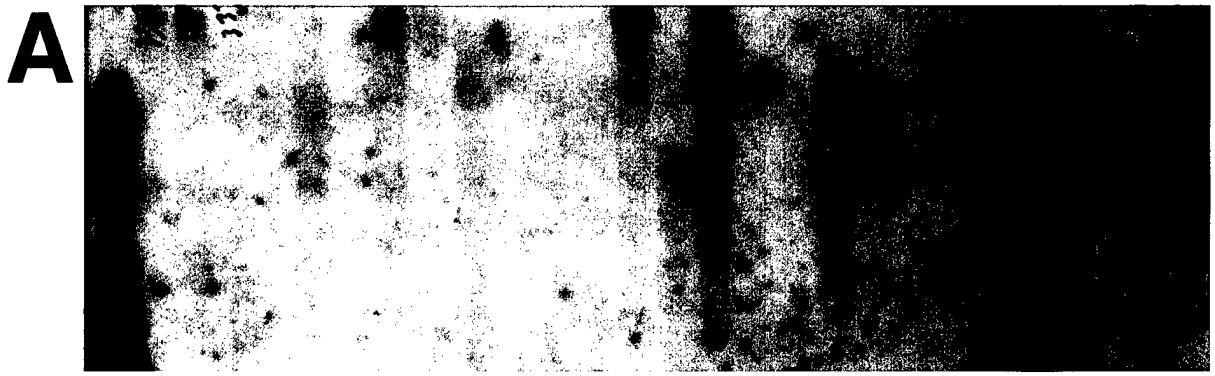
Panel D and E: Low and high power photomicroscopy of tumor 4395 histopathology (H+E stained section), showing an adenocarcinoma with abnormal gland formation and areas of necrosis.
Panel F: High power photomicroscopy of histopathology (H+E stained section) of 4395 mammary tumor transplanted into nude mouse, showing essentially the same cellular morphology as the original tumor (Panel E).

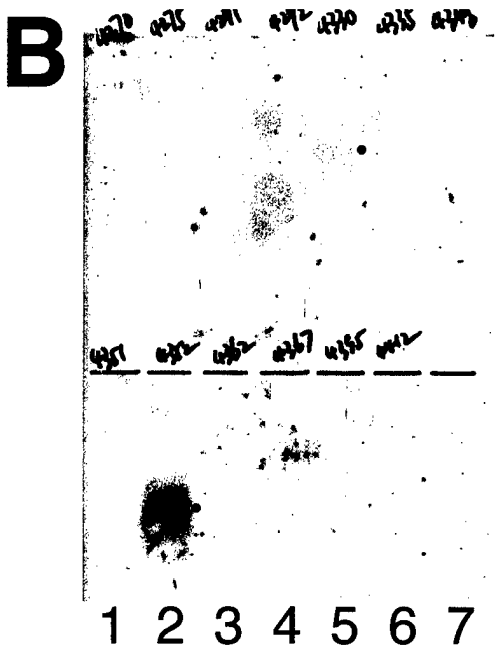
Figure 3A: Southern blot analysis of BACs 598K20 (lanes 1 and 2) and 598K21 (lanes 3 and 4) cut with HindIII (lanes 1 and 3) or PvuII (lanes 2 and 4) and hybridized with a probe for NDFα2A. Figure shows specific hybridization of the probe to clone 598K20 but not to 598K21.

3B. Kaplan-Meier plot of the disease-free survival of mice of three different types: FVB mice carrying the MMTV-c-*neu* transgene (coarse dash); virus negative C3H/eOuJ mice carrying the MMTV-c-*neu* transgene (solid line); and MMTV-positive negative C3H/eOuJ mice carrying the MMTV-c-*neu* transgene (fine dash).

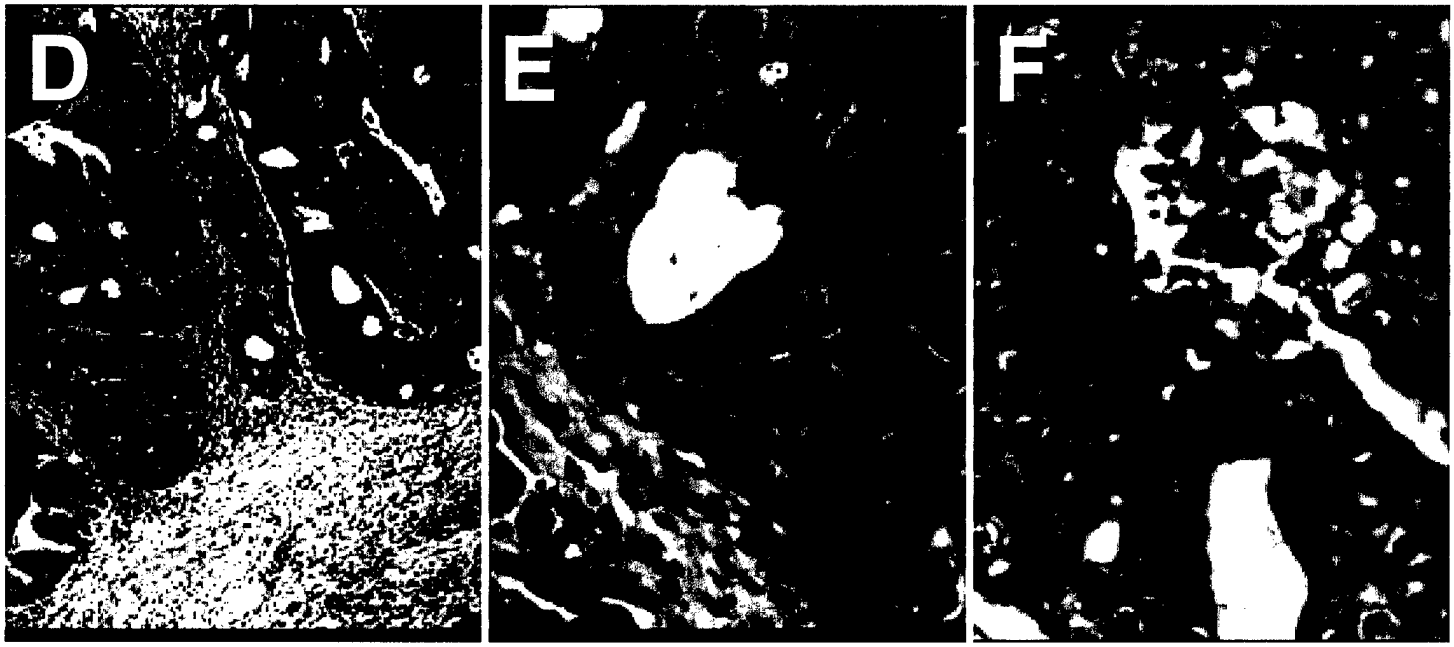
3C. Southern blot analysis of mammary tumor DNA for MMTV proviral insertions. Tumor DNA (10 μg) was cut with EcoR1, fractionated on agarose gels, blotted, and hybridized with a 1.2 kb BamHI probe comprising the MMTV *env* gene. The predicted presence (on the basis of the mating strategy and genotype analysis) of the MMTV and the MMTV-c-*neu* transgene is indicated above each lane.

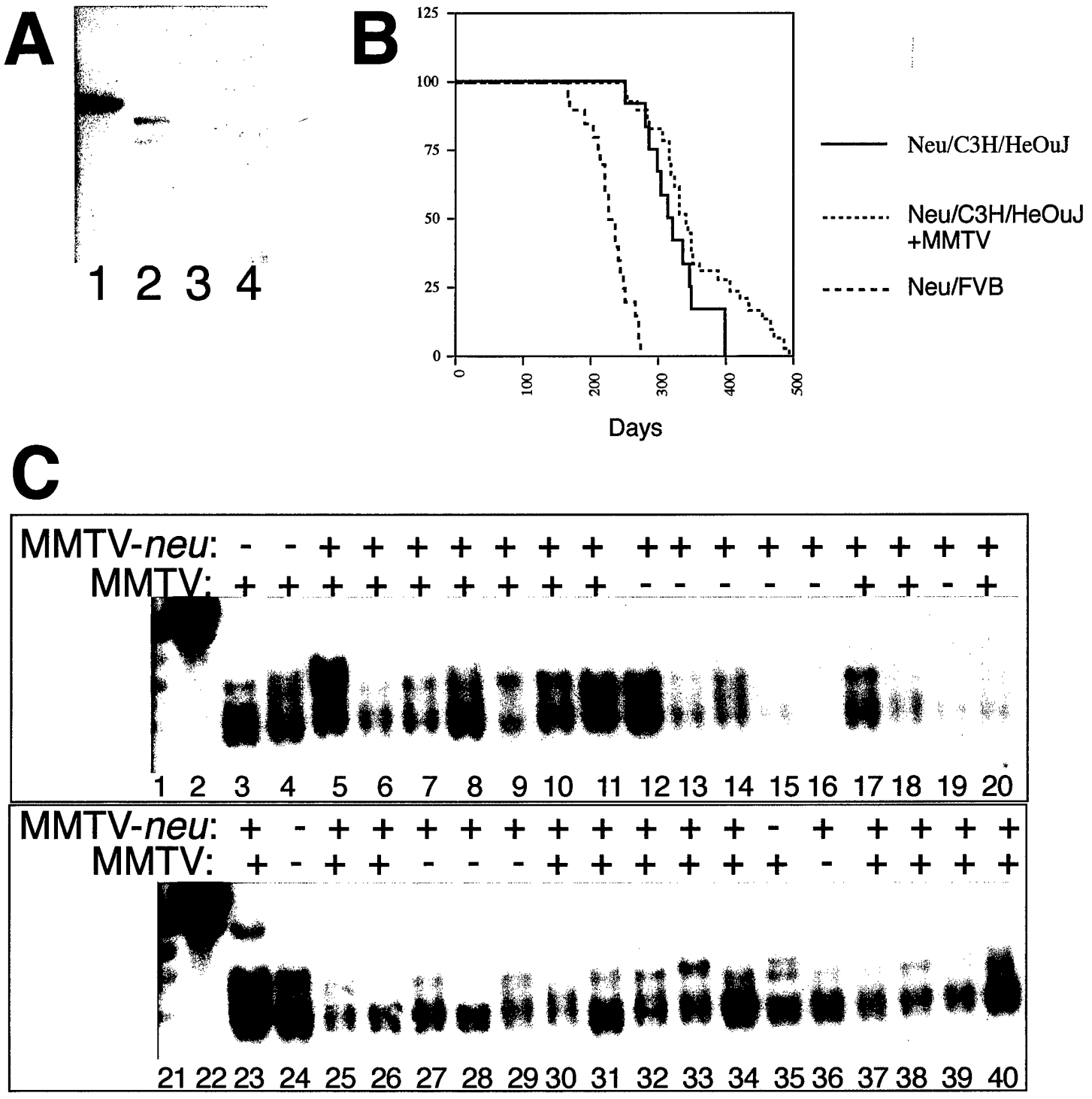

A
N
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
C
4481
D
4481
E
4483
F
non-transgenic





MMTV-TNT Mouse 4395: mammary tumor




A
1 2 3 4
B
125
100
75
50
25
0
0
100
200
300
400
500
Days
Neu/C3H/HeOuJ
Neu/C3H/HeOuJ +MMTV
Neu/FVB
C
MMTV-neu: - - + + + + + + + + + + + + + + + + +
MMTV: + + + + + + + + + - - - - - + + - +
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
MMTV-neu: + - + + + + + + + + + + + - + + + + +
MMTV: + - + + - - - + + + + + + - + + + + +
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40